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N-ACETYLATION OF THE GLUTAMATE RESIDUE OF INTACT GLUTATHIONE CONJUGATES IN RATS: A NOVEL PATHWAY FOR THE METABOLIC PROCESSING OF THIOL ADDUCTS OF XENOBIOTICS

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ABSTRACT:

We report herein the identification of a novel metabolic pathway that involves acetylation of the amino group of the glutamic acid residue of intact glutathione (GSH) conjugates of a series of compounds in rat hepatocytes and in rats in vivo. The "nonacetylated" as well as the "acetylated" GSH conjugates of the compounds in question were detected in rat hepatocyte incubations and in rat bile. These conjugates were characterized by online liquid chromatography-mass spectrometry on an ion-trap mass spectrometer as well as accurate mass measurements using a high-resolution quadrupole time-of-flight instrument. The accurate mass measurements on the molecular ions of nonacetylated and acetylated GSH adducts clearly revealed the addition of a mass equivalent to C_2H_2O in the latter conjugates. Furthermore, the collision-induced dissociation of the molecular ions of nonacetylated GSH adducts yielded fragment ions involving the loss of pyroglutamate (129 Da), which are typical of many GSH conjugates. For acetylated adducts, however, fragment ions resulting from a loss of 171 Da (equivalent to *N*-acetyl-pyroglutamate) were observed, indicating that acetylation had occurred on the glutamic acid residue of the GSH conjugates. An enzyme-catalyzed transacetylation process that utilized acetyl CoA as the acetyl donor, and resulted in the formation of the same acetylated adducts that were detected in rat hepatocytes and in rat bile, was identified in rat liver microsomes. This appears to be the first reported instance of *N*-acetylation of intact GSH conjugates in any species and represents a novel pathway of metabolic processing of thiol adducts of xenobiotics.

Glutathione (GSH[1]) conjugation is a prominent pathway of metabolism for many xenobiotics, especially the ones that are bioactivated to reactive electrophilic intermediates via oxidative metabolism (Parkinson, 2001). The GSH conjugates may either be excreted intact in bile or may undergo further processing via the so called "mercapturic acid pathway". The latter involves sequential cleavage of the glutamic acid and glycine residues of the GSH moiety via the enzymes γ-glutamyl transpeptidase (to form cysteinylglycine-*S*-conjugates) and aminopeptidase-M (to form cysteine-*S*-conjugates), respectively (Commandeur et al., 1995; Parkinson, 2001). This processing of GSH conjugates is believed to occur in the kidney because of a high activity of the above enzymes in this tissue. The resulting cysteine *S*-conjugates are then *N*-acetylated at the cysteine amino group via the cysteine *S*-conjugate *N*-acetyltransferase to form *N*-acetyl cysteine or mercapturic acid metabolites (Commandeur et al., 1995). This *N*-acetylation reaction can occur in the kidney itself or the cysteine *S*-conjugates may be transported back to the liver and acetylated in that tissue; the resulting mercapturic acid metabolites, however, are almost always excreted in urine (Inoue et al., 1982, 1984; Commandeur et al., 1995). Some recent studies have suggested the presence of at least one alternate pathway for the metabolic processing of GSH conjugates of xenobiotics. This pathway involves the conjugation of GSH, cysteinylglycine-, and cysteine *S*-conjugates with the γ-carboxylic acid moiety of glutamic acid to form extended peptide conjugates (Mutlib et al., 2000, 2002). Herein we describe the identification of another novel pathway for the metabolic processing of thiol adducts in rats that involves the *N*-acetylation of the glutamic acid residue of intact GSH conjugates.

[1] Abbreviations used are: GSH, glutathione; LC, liquid chromatography; MS, mass spectrometry; HPLC, high-performance liquid chromatography; CID, collision-induced dissociation; Q-Tof, quadrupole time-of-flight; amu, atomic mass unit(s); XIC, extracted ion chromatogram.

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Materials and Methods

Materials. Compounds **1** to **3** (Fig. 1) were synthesized within the Department of Medicinal Chemistry, Merck Research Laboratories (Rahway, NJ). A tritiated analog of **1** (specific activity 37 mCi/mg, purity >98.5%) was synthesized by Labeled Compound Synthesis, Department of Drug Metabolism, Merck Research Laboratories. A protein assay reagent kit was purchased from Pierce Chemical (Rockford, IL). All other chemicals were purchased from Sigma-Aldrich (St. Louis, IL) and were of reagent grade. All animal procedures performed were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee.

Incubations with Rat Hepatocytes. Hepatocytes were isolated from the perfusion of the whole male rat liver using a two-step perfusion procedure (Pang et al., 1997). After isolation, hepatocyte preparations that exhibited a viability of >80%, as determined by trypan blue exclusion, were suspended in Krebs-Ringer bicarbonate buffer (pH 7.4).

For hepatocyte incubations, compounds **1** to **3** (10 μM) were incubated at 37°C for 2 h with 4 ml of a freshly isolated hepatocyte suspension containing

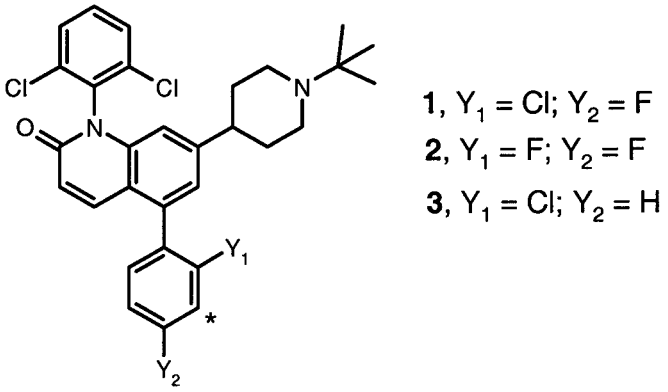

FIG. 1. *Structures of the five compounds discussed in the text.*

⋆ denotes the position of the tritium label in the radiolabeled analog of **1**.

one million cells/ml in Krebs-Ringer bicarbonate buffer. Incubations were conducted in stoppered flasks and were aerated with a 5% CO_2/95% O_2 gas mixture at hourly intervals. At the end of the 2-h period, reactions were quenched by addition of 4 ml of acetonitrile, and samples were centrifuged to separate the precipitated residue. Supernatants were concentrated by evaporation under nitrogen before analysis by LC-MS^n using an ion trap mass spectrometer.

In Vivo Metabolism Studies in Rats. Male Sprague-Dawley rats ($n = 3$) were anesthetized with sodium pentobarbital (Nembutal), and their femoral veins and bile ducts were cannulated with PE-10 tubing. Animals were allowed to recover for at least 48 h before experimentation. On the day of the experiment, rats were placed in metabolism cages, and pretreatment bile and urine samples were collected. Doses of **1** were prepared in an ethanol/PEG 400/water (2:2:6) solvent mixture. **1** was administered to rats intravenously via the femoral vein (2 mg/kg, 100 μCi/kg). Cumulative bile and urine samples were collected into tared containers at predetermined intervals for up to 72 h after dosing and frozen at −20°C before analysis. Radioactivity concentrations in bile and urine samples were measured using liquid scintillation counting. Bile samples were extracted with acetonitrile and concentrated via evaporation under nitrogen before LC-MS^n analysis.

Incubations with Rat Liver Microsomes. Male rat liver microsomes were prepared by standard differential centrifugation procedures (Raucy and Lasker, 1991). Livers from 40 naive male rats were pooled for microsome preparation. Microsomal protein concentrations were measured according to the manufacturer's instructions for the use of the protein assay kit.

1 (10 μM) was incubated at 37°C with rat liver microsomes (2 mg/ml protein) in 100 mM potassium phosphate buffer (pH 7.4) containing 10 mM magnesium chloride and an NADPH-regenerating system consisting of glucose 6-phosphate (10 mM), $NADP^+$ (1 mM), and glucuose-6-phosphate dehydrogenase (0.7 unit/ml). All microsomal incubations were also supplemented with 5-mM GSH. Reactions were started by the addition of the substrate and incubations were carried out for 2 h. To examine the acetylation of GSH conjugates formed in the above microsomal incubations, acetyl CoA (1 mM) was added to some incubations in the beginning and at the end of 1 h. Negative control incubations proceeded without the addition of acetyl CoA. Incubations without the NADPH-regenerating system were also conducted to serve as additional negative controls. Furthermore, to demonstrate that the observed acetylation of intact GSH conjugates is catalyzed by a microsomal enzyme, **1** (10 μM) was incubated with rat liver microsomes in the presence of an NADPH-regenerating system and 5 mM GSH for 2 h, and the incubates were filtered using 12,000 mol. wt. cutoff filters to remove the microsomal material. The filtered incubates containing the in situ generated GSH adducts of **1** were then incubated with acetyl CoA (1 mM) in the presence or absence of rat liver microsomes (2 mg/ml protein) for an additional 2 h. Negative control incubations included acetyl CoA either with or without boiled rat liver microsomes. To determine the subcellular localization of enzyme(s) catalyzing the acetylation of GSH conjugates, parallel incubations also were carried out in the presence of rat liver cytosol (2 mg/ml protein). At the end of the incubation time, the reactions were quenched with 2 ml of acetonitrile. Samples were vortex mixed, refrigerated to precipitate the protein, and centrifuged at 3000*g* for 15 min. The supernatant was concentrated by evaporation under nitrogen before analysis by LC-MS^n.

LC-MS^n Analysis. Chromatographic separation of metabolites was achieved on a Phenomenex Synergi Polar RP-80A reverse-phase HPLC column (25 cm × 4.6 mm, 4 μm; Phenomenex, Torrance, CA) using two PE Series 200 pumps and a PE Series 200 auto-injector (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). Mobile phase A consisted of 5 mM ammonium acetate and mobile phase B of a 70:30 acetonitrile/methanol mixture. Both mobile phases contained 0.1% formic acid. Metabolites of interest were eluted using a gradient profile in which the HPLC run began with 30% solvent B for the first 2 min, which was increased to 40% at 40 min, and then to 90% at 50 min using linear gradients. Solvent composition was held at 90% B until 60 min to flush the column before re-equilibration. Flow rate was 1 ml/min and the column effluent was split 1:5 between the mass spectrometer and the online radioactivity detector, respectively. Radioactivity detection was performed using an online radioactivity detector (β-RAM; IN/US Systems, Tampa, FL). The structural identification of metabolites was performed using a Finnigan LCQ DECA XP Ion Trap Mass Spectrometer (Thermo Finnigan, San Jose, CA) operated in positive ion electrospray mode. The spray voltage was set at 4.5 kV and the sample transfer tube temperature at 300°C. The mass spectrometer was operated in the data-dependent acquisition scan mode to simultaneously acquire full scan MS and MS^n (generally only up to MS^2) data. The relative collision energy for CID experiments was set at 45%.

Accurate Mass Measurements using a Q-Tof II Mass Spectrometer. The Q-Tof II mass spectrometer (Micromass, Inc., Cheshire, UK) had a minimum mass resolution of 6000 [measured as full width at half-maximum (FWHM)] during all experiments. The mass spectrometer was operated in positive ion electrospray mode and was calibrated on each experiment day using a mixture solution of polyethylene glycols with known exact molecular masses (approximate range 100-1000 Da). Electrospray capillary voltage and cone voltage were set at 3400 V and 40 V, respectively. Source and desolvation temperatures were 100 and 300°C, respectively. Nitrogen was used as the desolvation and nebulizing gas at flow rates of 400 and 50 l/h, respectively. Full scan Tof MS spectra were acquired for measurement of accurate masses of molecular ions of parent compounds and relevant metabolites. Chromatographic conditions were the same as those used for the LC-MS^n experiments described above, except that a Hewlett-Packard Series 1100 HPLC system (Hewlett Packard, Palo Alto, CA) consisting of two pumps, an autosampler, and a column oven was utilized. Leucine-enkephalin (1 μg/ml) was introduced into the column effluent at a rate of 5 μl/min via a T-joint to act as an internal mass calibrant (lock-mass). Acquisition and analysis of the data were performed using MassLynx software (version 3.5, Micromass Inc.).

Results and Discussion

At least three different groups of GSH adducts, with molecular ions at *m*/*z* 862, 860, and 904, were detected upon incubation of **1** with rat hepatocytes (Fig. 2). Figure 3 shows the MS^2 mass spectra obtained using CID of molecular ions of these three groups of GSH adducts, as well as the relevant fragment ion assignments; the spectra of the two adducts within each group were identical. The mass of the molecular ion of GS1a and GS1b suggests an addition of 305 Da to the mass of **1**, which corresponds to the addition of one GSH molecule. The MS^2 spectrum of adducts GS1a and GS1b (Fig. 3A) shows a characteristic loss of 129 amu (corresponding to the pyroglutamate residue of the added GSH moiety; Baillie and Davis, 1993) to form an ion at *m*/*z* 733, which is consistent with this metabolite being a GSH adduct. The fragment ions at *m*/*z* 533 and 589 apparently correspond to the cleavage of the C–S bond of the cysteine residue either with or without the concurrent loss of the *t*-butyl moiety, thus leaving a free thiol substituent on the core structure of **1**. All of these GSH adducts were formed in trace amounts only in rat hepatocyte incubations, and therefore, it was not possible to isolate these in enough quantities for determination of exact regio-chemistry of GSH addition using NMR. However, we have evidence from our work on some other close

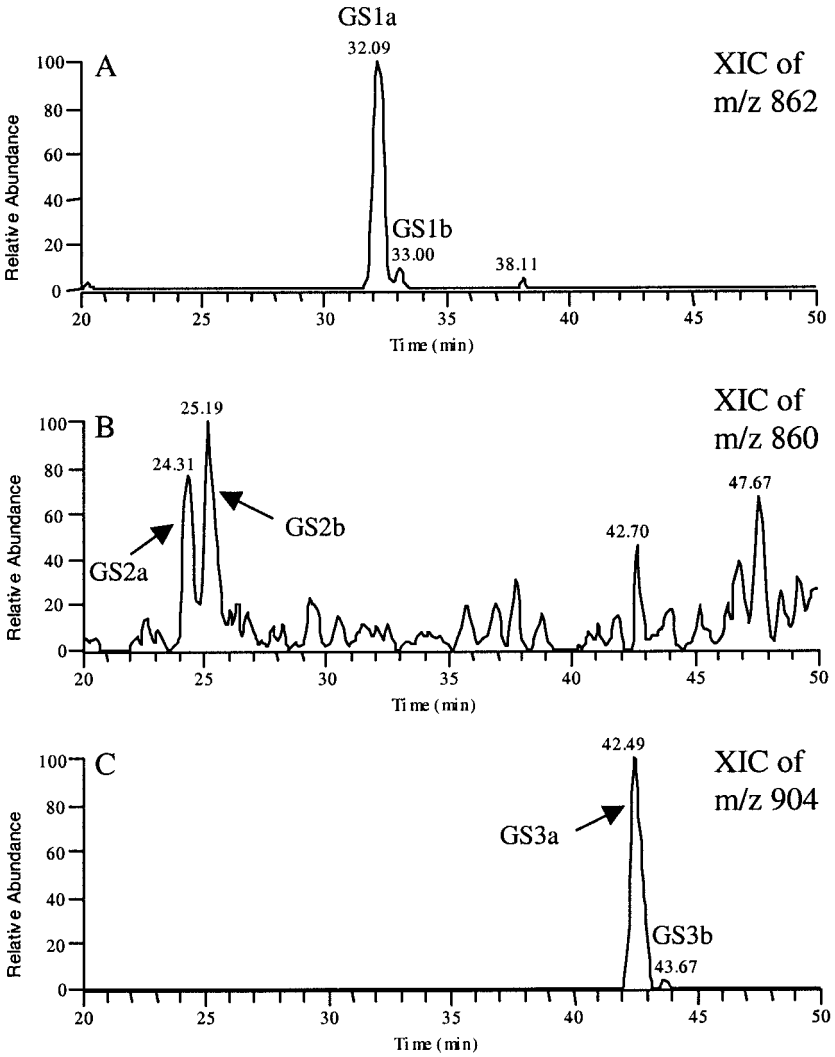

FIG. 2. *Extracted ion chromatograms (XIC) of GSH adducts of* **1** *detected in rat hepatocyte incubations.*

analogs of **1** that the "bottom" halo-substituted aryl ring is the site of bioactivation and GSH addition for these compounds (also see below).

The *m/z* of the molecular ion of adducts GS2a and GS2b indicates an addition of 303 Da to the mass of **1**. Such adducts typically result from a mechanism involving epoxidation of the aromatic ring adjacent to the fluorine substitution, followed by the addition of GSH and loss of HF, such that the original fluorine subsituent is replaced with an -OH moiety (Fig. 5; Park et al., 2001; Parkinson, 2001; Samuel et al., 2003). The MS^2 spectrum of GS2a and GS2b (Fig. 3B) is very similar to that seen for GS1a and GS1b except for the 2-amu difference in all fragment ions involving the core structure of **1.** This is consistent with the replacement of fluorine substitution with an OH moiety in the GS2a and GS2b structure and provides additional evidence that the "bottom" aryl ring of **1** is the site of bioactivation and GSH addition in rat hepatocytes.

The nominal mass of the molecular ions of adducts GS3a and GS3b indicates the addition of 347 Da to the mass of **1**, which can be interpreted as the addition of one GSH (305 Da) and one acetyl (42 Da) moiety. To further substantiate this interpretation, accurate mass measurements were performed on the molecular ions of the parent compound **1**, GS1a and GS1b, and GS3a and GS3b adducts using a high-resolution Q-Tof II mass spectrometer. The accurate mass data, the suggested elemental composition, and deviations of the measured mass from the theoretically calculated mass are presented in Table 1. The small deviations (<5 ppm) observed in all cases lend support to the suggested elemental composition presented in Table 1 for these compounds. The indicated elemental composition of GS1a and GS1b is consistent with these being the products of addition of one molecule of GSH to the parent **1**. Furthermore, the comparison of the suggested elemental compositions of GS1a and GS1b versus GS3a and GS3b suggests a difference in mass corresponding to C_2H_2O (Table 1); this supports the interpretation that GS3a and GS3b represent acetylated analogs of GS1a and GS1b or regio-isomers thereof. Interestingly, the MS^2 fragmentation of GS3a and GS3b involved a characteristic loss of 171 amu (instead of 129 in GS1a and GS1b) to form the same fragment ion that was observed for GS1a and GS1b at *m/z* 733 (Fig. 3C). This indicates that the acetylation has occurred on the amino group of the glutamate residue of the adduct (Fig. 3C). Many other fragment ions observed in the MS^2 spectrum of GS3a and GS3b were similar to those observed for GS1a and GS1b and support the interpretation that the core structure of **1** was unaltered in these adducts.

Similar adducts were observed for compounds **2** and **3** also upon incubation with rat hepatocytes, and their MS^2 spectra as well as accurate mass measurements were in line with the observations made for compound **1** above (data not shown).

When a 2 mg/kg dose of **[^{3}H]1** was administered intravenously to

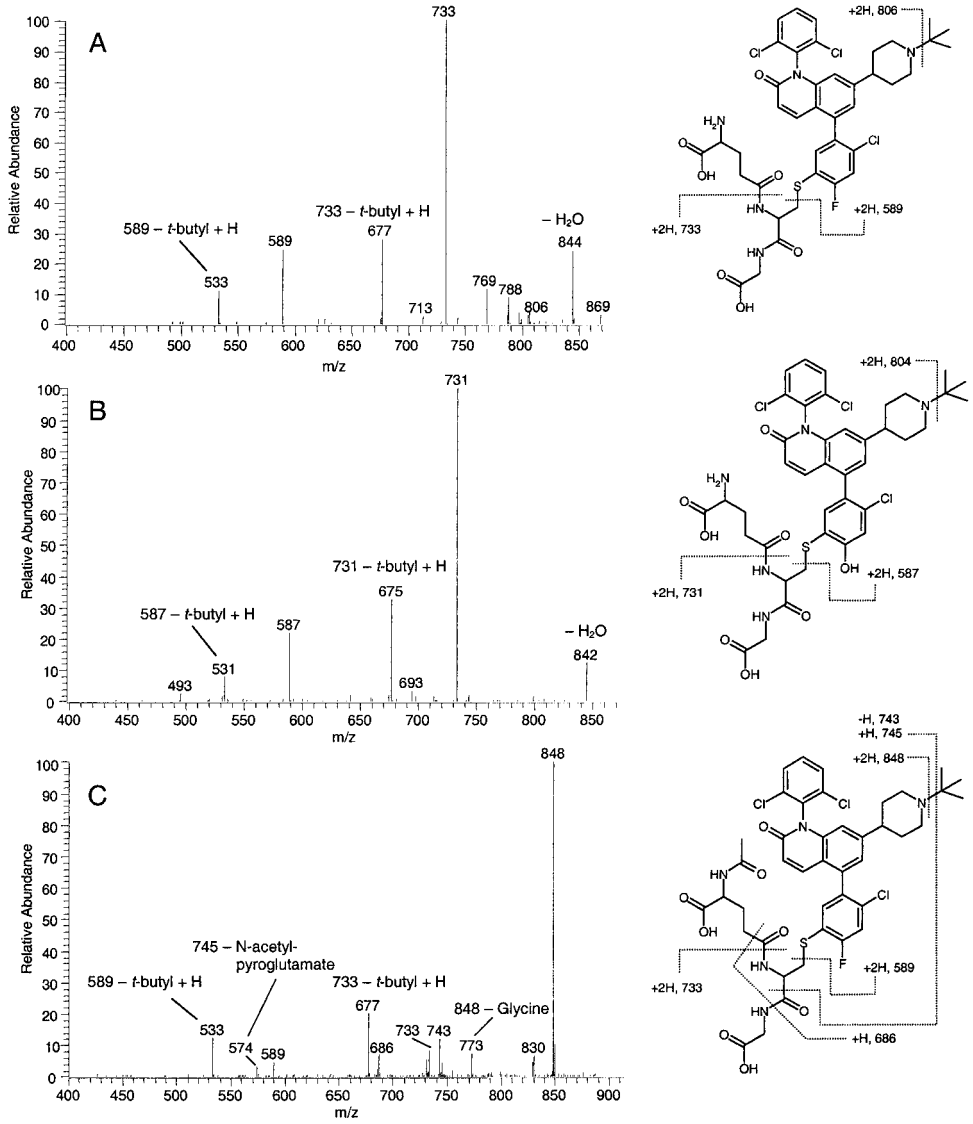

FIG. 3. *MS^2 spectra of GSH adducts of* **1** *with molecular ions at m/z 862 (GS1a and GS1b) (A), m/z 860 (GS2a and GS2b) (B), and m/z 904 (GS3a and GS3b) (C) that were detected in rat hepatocyte incubations.*

The exact regio-chemistry of GSH addition on the molecule is not known, and the structures drawn are for illustration only. These same adducts were detected in rat bile after **[^{3}H]1** administration as well.

TABLE 1

Accurate masses of the molecular ions (MH^+) of the GSH- and N-acetyl-GSH adducts of **1** *in rat hepatocytes*

Accurate mass measurements were obtained using high-resolution time of flight mass spectrometry on a Q-Tof II mass spectrometer. The suggested elemental composition and deviations from the calculated masses are also presented.

Compound/Metabolite	Measured Mass of MH^+ Ions	Suggested Elemental Composition	Deviation from the Calculated Mass	
			ΔmDa	Δppm
	Da			
1 Parent	557.1309	$C_{30}H_{29}N_2OFCl_3^+$	+2.0	−3.6
GS1a and GS1b	862.1981	$C_{40}H_{44}N_5O_7FSCl_3^+$	−3.0	−3.5
GS3a and GS3b	904.2139	$C_{42}H_{46}N_5O_8FSCl_3^+$	+2.2	+2.4

mDa, milli-Daltons.

bile duct-cannulated rats, ~65% of the administered radioactivity was recovered in bile within 72 h after dosing. Of that 65%, a pyridinium metabolite formed via aromatization of the *t*-butyl piperidine, and glucuronic acid conjugates of multiple hydroxylated metabolites of **1** accounted for ~75% and were the major components. In addition, small amounts of the parent compound (5%) and unconjugated mono- (5%) and dihydroxylated metabolites (5%) were also present. All three groups of GSH adducts detected in rat hepatocyte incubations were identified in rat bile as well and collectively accounted for ~10% of the total radioactivity excreted in bile. The composition of the remaining 35% of the radioactivity that was not recovered during the course of these studies remains unknown. These data indicate that the novel pathway that leads to the *N*-acetylation of intact GSH adducts at the glutamic acid residue is operational in rats in vivo as well as in vitro.

The GSH adducts GS1a and GS1b of **1** identified above in rat hepatocyte incubations and in rat bile were also formed in rat liver microsomal incubations that were supplemented with 5 mM GSH, although the relative proportions of the two isomers were somewhat different relative to those observed in hepatocytes and in rat bile (Fig. 4A, upper panel). As expected, however, their corresponding acety-

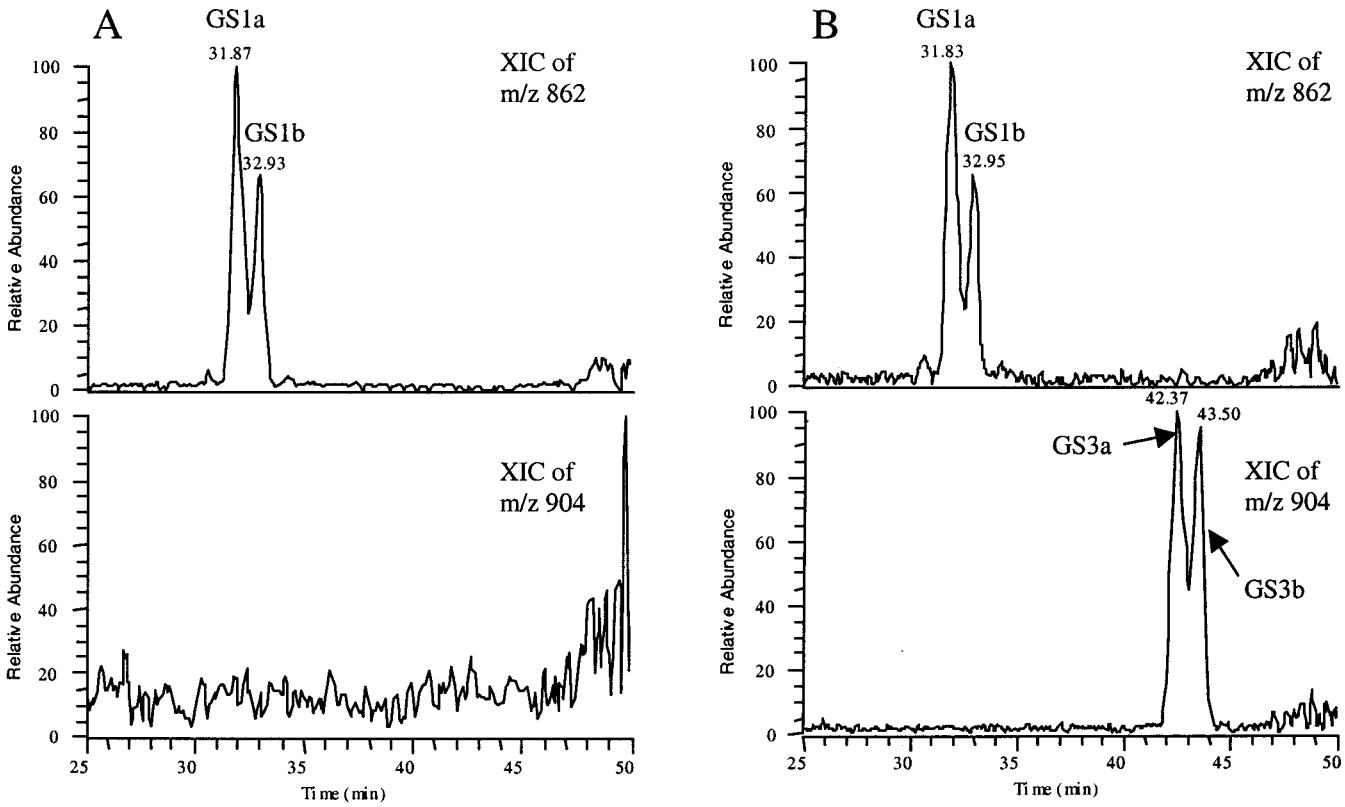

FIG. 4. *Extracted ion chromatograms (XIC) of GSH adducts of* **1** *in rat liver microsomal incubations conducted either in the absence (A) or presence (B) of 1 mM acetyl CoA and supplemented with 5 mM GSH.*

The upper panel is XIC at *m/z* 862 (corresponding to GS1a and 1b) and the lower panel is at *m/z* 904 (corresponding to GS3a and 3b) in both A and B.

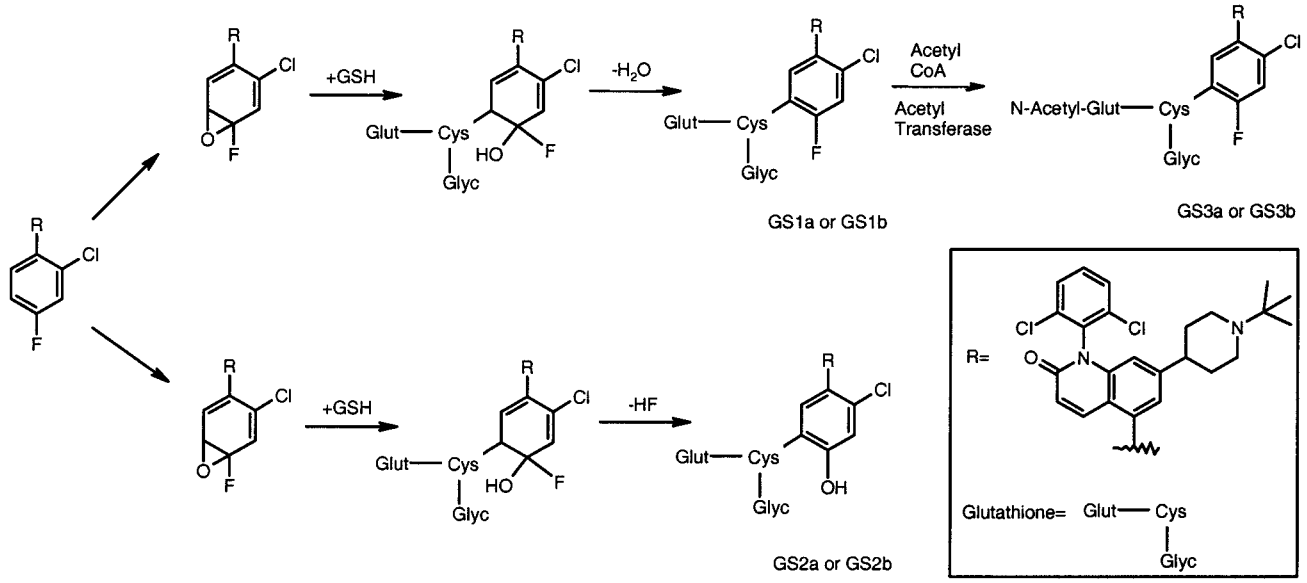

FIG. 5. *Postulated mechanisms for the formation of various GSH adducts of* **1** *that were identified in rat hepatocyte incubations and in rat bile.*

The exact regio-chemistry of GSH addition in these adducts is unknown, and multiple other regio-isomers are possible. The drawn structures are for illustrative purposes only.

lated analogs GS3a and GS3b were not present in these incubations (Fig. 4A, lower panel). Interestingly, when 1 mM acetyl CoA was added to these microsomal incubations, GS3a and GS3b were readily detectable (Fig. 4B, lower panel). These data indicate that acetyl CoA acts as an acetyl donor for the acetylation of GS1a and GS1b to GS3a and/or GS3b in rat liver microsomal incubations (Fig. 5). When rat liver microsomal incubates of **1** containing GS1a and GS1b were filtered to remove the microsomal material and were further incubated with acetyl CoA in the presence or absence of active rat liver microsomes, boiled rat liver microsomes, or rat liver cytosol, the acetylated

conjugates GS3a and GS3b were detected only in incubations containing active rat liver microsomes. Thus, the transacetylation of GS1a and GS1b to GS3a and GS3b appears to be catalyzed by an enzyme present in rat liver microsomes. The identity of the enzyme(s) catalyzing this novel transacetylation reaction remains to be determined. Previous studies indicate that cysteine *S*-conjugate *N*-acetyltransferase, which is responsible for the acetylation of cysteine *S*-conjugates to the corresponding mercapturic acids (*N*-acetyl cysteine conjugates), is also a microsomal enzyme, with its active site located toward the cytoplasmic surface, and the highest activities of this enzyme are present in the liver and the kidney (Okajima et al., 1984; Commandeur et al., 1995). It is possible that either the same enzyme or a closely related member of the same family is responsible for the acetylation of intact GSH adducts at the glutamic acid residue, as observed in the current studies.

To our knowledge this is the first reported instance of the acetylation of intact GSH adducts of xenobiotics in any species. This metabolic route, in combination with the recently discovered "glutamic acid pathway" that results in the formation of extended peptide adducts (Mutlib et al., 2000, 2002), represents examples of alternate metabolic processes for the handling of xenobiotic-GSH conjugates relative to the more common mercapturic acid pathway. The biological significance of these pathways of processing of GSH conjugates, and whether these metabolic processes exist in species other than the rat, is not known at present. Furthermore, it remains to be determined whether these "acetylated" GSH conjugates can serve as substrates for γ-glutamyl transpeptidase and be converted to the corresponding cysteinyl-glycine conjugates, as is known to occur for numerous other "nonacetylated" GSH adducts. It appears, however, that multiple routes are likely available for the sequential processing of thiol conjugates of xenobiotics in mammalian species, and these will continue to be discovered with recent innovations in sophisticated analytical methodologies.

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